TWO LABORATORY CASE STUDIES ON THE ORAL TOXICITY TO CALVES OF THE FRESHWATER CYANOPHYTE (blue-green alga) *ANABAENA FLOS-AQUAE* NRC-44-1

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Introduction

Poisonings by toxic waterblooms of freshwater cyanophytes (blue-green algae) often cause a considerable economic loss when livestock are involved. Three toxic genera, *Microcystis*, *Aphanizomenon* and *Anabaena*, have been implicated in these poisonings. Of these genera, the species *Anabaena flos-aquae* has received the most thorough investigation (2, 5). This cyanophyte often forms thick wind-concentrated surface blooms or scums in eutrophic lakes and sloughs in Western Canada during the summer months. Some of these blooms are toxic and when a critical dose is ingested by livestock or other animals deaths can occur from 10 to 20 minutes, up to several hours. Alberta and Saskatchewan, in particular, have had intermittent but repeated cases of cyanophyte poisonings, many of which have been recorded by Schwimmer *et al.* (11) and more recently for Saskatchewan by Hammer (6). Some recent cases which we have investigated include the following:

1. Disney Lake near Strathmore, Alberta, June 1972. Three calves died due to poisoning by *A. flos-aquae* (Alberta Department of Agriculture, Veterinary Services Division, Laboratory Services, No. 72-3305).
2. Beaverhill Lake near Mundare, Alberta, September 1972. Fifteen head of cattle died due to *A. flos-aquae* (Alberta Department of Agriculture, Veterinary Services Division, Laboratory Services, No. 72-4273).
3. Unnamed lake west of Bruno, Saskatchewan, July 1975. Thirty-four head of cattle died from algae poisoning, with the veterinary report (Provincial Veterinary Laboratory Report, Regina, Saskatchewan Pathology No. 75-384) indicating that *Microcystis* sp. was the dominant cyanophyte present at the time. Samples of blooms collected from this lake about one month later and studied by this laboratory, were found to have both toxic *Microcystis aeruginosa* and toxic *Anabaena flos-aquae* colonies in high concentrations.

Signs of poisoning by *A. flos-aquae* in animals usually include staggering, muscle fasciculations, gasping respiration and convulsions. Death of such animals occurs anywhere from five to ten minutes in laboratory mice and up to several hours in livestock after ingestion of a lethal bolus. The short survival times are indicative of a neuromuscular poison and this has been verified using laboratory rats (2). These toxinological tests with rats showed that death was primarily caused by respiratory arrest since, with artificial respiration, normal body functions such as ECG, and blood pressure could be maintained for periods up to at least eight hours.

The purpose of these case studies with calves was to observe the signs of poisoning and estimate the oral minimal lethal dose (MLD) under conditions of controlled dosage. Another purpose was to test the possibility that dosing with slightly more than the oral MLD it might be possible and practical with artificial respiration to maintain essential body functions long enough for natural detoxification to occur and normal neuromuscular responses to become reestablished.

Materials and Methods

Anabaena flos-aquae NRC-44-1 was grown for two weeks in aerated ASM-1-TR medium in 8-l Pyrex bottles at 21°C with continuous illumination of 5500 lux provided by four cool-white fluorescent lamps. Mature cultures were acidified (pH 4) and concentrated with a vacuum still at 45°C. The concentrates were lyophilized and the dried product pooled, ground in a mortar, bioassayed for toxicity (MLD, intraperitoneal mouse (i.p.m.) = 60 mg/kg body weight) and stored in dark bottles with screw caps at 5°C until needed. The doses used were prepared just prior to adminis-

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TABLE I

EXPERIMENTAL PROTOCOL AND PRINCIPAL OBSERVATIONS WITH TIME FOLLOWING THE ORAL DOSING OF A 73 kg WEANED MALE CALF WITH A SUSPENSION OF LYOPHILIZED *Anabaena flos-aquae* NRC-44-1. ARTIFICIAL RESPIRATION WAS APPLIED SUBSEQUENT TO RESPIRATORY ARREST. BLOOD SAMPLES WERE TAKEN AND DRUGS ADMINISTERED *via* THE JUGULAR VEIN. BLOOD PRESSURE WAS MEASURED AT THE FEMORAL ARTERY

Time (elapsed) min	Event or Observation	Blood $P O_2$, mm Hg	Blood $P CO_2$, mm Hg	pH
0	Blood sample #1. Dose of 120 g (= 27 × MLD i.p.m.). Cells were suspended in 700 ml water and rinsed into stomach with 300 ml water	25	35	7.20
4	Staggers, convulsions, muscle fasciculation, breathing abdominal (animal down). Intubated, artificial respiration begun.			
32	Blood sample #2	25	35	7.13
57	Blood sample #3	25	37	7.18
61	1 ml (10 mg) of edrophonium chloride injected. No antagonistic effect observed			
119	ECG normal, blood pressure, 100 mm Hg (average of systole-diastole)			
222	No indication of any return of muscle control. Experiment terminated			

Autopsy showed all organs apparently normal. Microscopic examination of contents in reticulorumen, omassum and abomassum showed fragmented *Anabaena flos-aquae* present in all stomachs, mixed with other feed.

tration by suspending a weighed amount of lyophilized powder in a measured volume of water, using a magnetic stirrer.

The calves used were obtained from the University of Alberta Farm. The experiments were conducted using the facilities of the University's Surgical Medical Research Institute (SMRI). The calves had been weaned about two weeks prior to the tests so that the rumen flora would be more similar to that of older animals on a grain and hay diet. The animals were starved overnight prior to use. The doses of lyophilized *Anabaena flos-aquae* NRC-44-1 chosen were based initially on oral minimum lethal dosages (MLD's) in rats and mice which were 1500 and 1800 mg/kg body weight, respectively. The calves were dosed *via* a stomach tube into the reticulorumen. The total bolus of *A. flos-aquae* NRC-44-1 suspension and water used for rinsing was about one litre. Once the animal collapsed from respiratory arrest it was intubated and placed on a "bird" Mark 2 respirator. Artificial respiratory rates and volumes were adjusted to maintain blood gases at about $PO_2 = 25 \pm 5$ mm Hg, $PCO_2 = 30 \pm 5$ mm Hg and a pH of 7.2 ± 0.2. Blood samples were taken at regular intervals from the jugular vein. Electrocardiogram (ECG) was recorded from the equivalent of human Lead II (9). These leads were to the four limbs (right hind leg ground) and a fifth to the central chest area. Blood pressure was measured by a transducer attached to the femoral artery. Both ECG and blood pressure outputs were attached to a Hewlett Packard (HP) carrier amplifier with recordings done on a HP-model 775417-4-channel physiograph.

RESULTS

The first calf dosed was a male Black Angus weighing 73 kg (ID number 7406). He was six weeks old and had been weaned at four weeks. The animal was dosed with 120 g of lyophilized NRC-44-1. This dose, 1644 mg/kg, was equal to approximately 27 MLD's (i.p.m.).

Results from this experiment (Table I) suggested that the oral MLD for calves had been exceeded because the onset of complete muscle paralysis was more rapid than expected on the basis of experiments with other animals. It was estimated that the oral MLD was more likely about one-half to one-third of that administered in this first case. At 61 min an anticholinesterase, edrophonium chloride, was administered but it had no apparent antagonistic effect on the toxin-induced neuro-muscular blockade. During the 222 minutes of the experiment, very little, if any, detoxification by the animal occurred. An autopsy showed all organs apparently normal. Fragmented *Ana-*

TABLE II

EXPERIMENTAL PROTOCOL AND PRINCIPAL OBSERVATIONS WITH TIME DURING AND FOLLOWING A SEQUENTIAL SUBLETHAL ORAL DOSING OF A 42.5 kg WEANED MALE CALF WITH A SUSPENSION OF LYOPHILIZED *Anabaena flos-aquae* NRC-44-1. ARTIFICIAL RESPIRATION WAS MAINTAINED AS REQUIRED, SUBSEQUENT TO RESPIRATORY ARREST. BLOOD SAMPLES WERE TAKEN AND DRUGS ADMINISTERED *via* THE JUGULAR VEIN

Time (elapsed) min	Event or Observation	Blood $P O_2$	Blood $P CO_2$	Blood pH
0	Blood sample. Dose #1 given—6.3 g (= 2.5 × MLD i.p.m.)	31.8		7.30
27	Blood sample #2	31.5	30.5	7.43
30	Dose #2—6.3 g (total = 5 × MLD i.p.m.)			
33	Muscle fasciculations in shoulder			
40	Muscle fasciculations in limbs			
43	Animal collapsed but still breathing			
48	Some paralysis (ptosis) of upper eyelid			
53	Blood sample #3	30	32.5	7.38
58	Dose #3. 3.15 g (total = 6.25 × MLD i.p.m.)			
90	Dose #4. 6.3 g (total = 8.75 × MLD i.p.m.)			
95	Heart rate 78/min, respiration 15/min Absence of definite response to last two doses indicated tachyphylaxis			
275	Respiratory collapse—animal placed on respirator			
375	I.V. jugular saline drip (0.9%) started to prevent dehydration of the animal			
472	10 mg edrophonium chloride (i.v.) caused some convulsive breathing but nothing permanent			
490	Five percent dextrose saline drip started (i.v.)			
496	0.25 mg neostigmine (i.v.) no permanent effect on breathing			
913	Animal regained normal respiration rate (33/min) and a heart rate of 100/min. No resumption of peripheral muscle control			
1308	Blood sample #8	22.8	39.5	7.35
1483	Respiratory collapse—animal intubated again			
1700	No indication of return of peripheral muscle control. Experiment terminated			

Autopsy showed all organs apparently normal. Microscopic examination of contents of reticulorumen, omassum and abomassum showed small amounts of fragmented *Anabaena flos-aquae* in all stomachs and in the small intestine. Urinary function of animal was normal after a dextrose-saline drip was administered (i.v.)

baena flos-aquae filaments were found in all three stomachs but concentrated in the reticulorumen and mixed with other feed.

To better estimate the oral MLD for calves and to check the possibility that at, or slightly more than, an MLD, a period of artificial respiration would provide time for detoxification to occur and the animal to regain muscle control, a second calf was tested. This calf was a male Black Angus (ID number 7411), five weeks, four days old, weighing 42.5 kg. He had been weaned at four weeks of age. For this experiment the animal was given a series of sublethal oral doses of the aqueous *Anabaena* suspension over a period of 90 min. On the basis of the first experiment the oral MLD was estimated at about ten times the MLD (i.p.m.). The sequential dose levels chosen were, therefore, 2.5, 2.5, 1.25 and 2.5 (total to 8.75 × MLD i.p.m.). It was hoped that in this way the MLD could be approached and just exceeded and that natural detoxification would occur during the subsequent period of artificial respiration. The experimental protocol and principal observations are outlined in Table II.

The results and conclusions of the second experiment may be summarized as follows:

1. The oral MLD for calves is between six and eight times the MLD (i.p.m.). This was concluded on the basis that the second calf collapsed with respiratory arrest after a total of 8.75 times the MLD (i.p.m.) was given. Since this dosage administered in sequential sublethal amounts, produced signs of tachyphylaxis (resistance to sequential sublethal dosages) it was likely that the acute MLD had again been exceeded.
2. Artificial respiration restored normal heart rate, blood pressure, blood gases and pH after respiratory collapse caused by the toxin. Sufficient natural detoxification did not occur during approximately 30 hours of artificial respiration to allow return of peripheral muscle control.
3. The anticholinesterases, edrophonium and neostigmine, which are effective in preventing curare or curare-like competitive postsynaptic block (7) could not sufficiently antagonize the toxin although some transient muscle twitches were seen after administration of these drugs.
4. Autopsy again showed that the cyanophyte filaments were distributed throughout the digestive tract of the calf.

Discussion

The oral MLD estimated for calves on the basis of these two case studies is within the range that animals in the field could be expected to consume in one drinking. This is based on a typical near-shore concentrated waterbloom of 20 mg dry wt/ml with an MLD (i.p.m.) of 60. This would mean that a 60 kg calf would require only about 1080 to 1440 ml of such a waterbloom to cause death (oral MLD = 360 to 480).

Since certain vital body functions were satisfactorily maintained during the prolonged period of artificial respiration it appeared that the toxin acted primarily as a peripheral neuromuscular blocking agent, although secondary effects on the central nervous system could not be ruled out. At this time we have not found an antagonist for the toxin since the anticholinesterases used, produced only a temporary potentiation of acetylcholine present in the nerve-muscle junction. Because of the rapid action of the toxin it is presumed that most of the toxin was absorbed into the blood while the cyanophyte cells were in the reticulorumen. The anatomy of the various compartments and the papillae of the ruminant digestive system were indicative of animals on a hay and grain diet (3). Based on these two case studies with calves, it was felt that the oral MLD range and signs of poisoning observed for the toxin could be related to dairy or beef cattle who are poisoned by toxic *Anabaena flos-aquae* (Type *a*) blooms in the field containing a toxin-like strain NRC-44-1.

Other work on the exotoxin (now called anatoxin-a) produced by *Anabaena flos-aquae* NRC-44-1 has shown it to be a potent depolarizing neuromuscular blocking agent that is rapidly absorbed by the oral route (2), (Carmichael, unpublished data). There are two other cyanophytes, *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*, which cause death of animals. The toxin of *Microcystis aeruginosa* NRC-1 is a cyclic polypeptide endotoxin called microcystin (1, 8, 10). The principal effects of microcystin when tested on laboratory and domestic animals were convulsions and visible abnormalities in the blood supply of the tissues (8). The peripheral circulatory system appeared drained of blood, causing anemia of ears, eyes and tail. The liver showed the most striking change, having a concentration of blood and general enlargement. It was often mottled with punctate hemorrhages bordered by yellowish-brown degeneration (8). The toxin of *Aphanizomenon flos-aquae* appears to be a neuromuscular endotoxin causing death by respiratory arrest (4).

The toxinological tests to date on *Anabaena flos-aquae* have largely involved axenic clone NRC-44-1. Using this toxic clone (single filament isolate) we have found that oral MLD's vary between animal species tested, in the following sequence goldfish < duck < calf < pheasant < rat < mouse. More recent isolations from other toxic waterblooms dominated by or containing *Anabaena flos-aquae* have shown that there are other toxic strains in nature (Carmichael, unpublished data). These other toxic strains also have neuromuscular properties and can be distinguished by their MLD's, signs of poisoning and survival times. Any complete understanding of cyanophyte poisonings to livestock will have to consider what these different toxin types do individually and in combination.

Summary

Two male, six week old calves were given lethal doses of a lyophilized culture of toxic *Anabaena flos-aquae* NRC-44-1 in aqueous suspension by stomach tube into the reticulorumen. The first calf developed muscle

fasciculations and loss of muscle coordination within four minutes of dosing and collapsed from respiratory arrest within seven minutes. Intubation and artificial respiration restored normal heart rate, electrocardiogram, blood pressure, blood gases, and pH. Normal respiration did not, however, return within four hours. A second calf given sequential doses showed similar effects as calf number one and collapsed from respiratory arrest three hours after administration of the final bolus. Artificial respiration was maintained for 30 hours with some intermittent but no permanent resumption of spontaneous respiration. On the basis of these experiments it was concluded that calves, and most likely other livestock, die from paralysis of respiratory muscle when enough of a toxic bloom of *A. flos-aquae*, producing a toxin like NRC-44-1, is consumed. The oral minimum lethal dose (MLD) is estimated at 420 mg/kg body weight. This means that a 60 kg calf could die after consuming about 1.2 litre of a surface-concentrated bloom of toxic *A. flos-aquae* (Type a) like strain NRC-44-1.

Résumé

Les auteurs ont administré à deux veaux mâles et âgés de six semaines, des doses léthales d'une suspension aqueuse d'une culture lyophilisée de l'algue toxique *Anabaena flos-aquae* NRC-44-1; ils utilisèrent à cette fin un tube oesophagien qu'ils firent pénétrer jusqu'à la jonction réseau-rumen. Le premier veau développa des fasciculations musculaires et une perte de coordination musculaire, en dedans de quatre minutes après l'administration de l'algue et s'effondra à la suite d'une syncope respiratoire, trois minutes plus tard. L'intubation et la respiration artificielle permirent un retour à la normale des battements cardiaques, de l'électrocardiogramme, de la pression sanguine, des gaz du sang et du pH. La respiration ne redevint toutefois normale qu'au bout de quatre heures. Un autre veau, auquel on avait administré des doses successives de cette algue, manifesta des signes analogues à ceux du premier veau et développa une syncope respiratoire, trois heures après l'administration du dernier bol. On lui prodigua la respiration artificielle, durant 30 heures; ceci lui permit de respirer de façon spontanée, par intervalles, mais non de façon constante. À la suite de ces expériences, les auteurs ont conclu que les veaux et vraisemblablement les autres animaux domestiques, meurent d'une paralysie des muscles respiratoires après avoir ingéré une quantité suffisante de l'algue en fleur toxique. A. *flos-aquae*, laquelle élabore une toxine similaire à NRC-44-1. La dose léthale minimale orale se situerait à 420 mg/kg de poids vif. Il s'ensuit qu'un veau pesant 60 kg pourrait mourir après avoir consommé environ 1.2 litre de l'algue fleurie et toxique *A. flos-aquae* (type a), concentrée à la surface d'un lac, et similaire à la souche NRC-44-1.

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